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13. ABSTRACT The following properties of <u>Chlamydia</u> are reviewed: Size of the genome, functions reflecting adaptation to intracellular environment, phenotypic and genotypic variation among strains of <u>Chlamydia</u> . Hypothetical step in the evolution of <u>Chlamydia</u> and the role of "genetic drift" are discussed.			

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Evolution of *Chlamydia**

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A discussion of the evolution of *Chlamydia* implies that we know a great deal about a large number of these organisms. Although there are still important gaps in our knowledge, the agent of meningopneumonitis (MN, *C. psittaci*) has been studied systematically in several laboratories. The information that we have on its structure, chemical composition, developmental cycle, enzymatic activity in an extracellular as well as in an intracellular environment, and on its interaction with the host cell is far greater than what we know of rickettsiae, non-cultivable mycobacteria, and most of the cultivable pathogens. In fact, we may consider the MN agent as the *Escherichia coli* of host-dependent bacteria. Excellent fundamental work has been done with a number of other established strains of *C. psittaci* and *C. trachomatis*. The least information is available on isolates that have not yet been adapted to yield large harvests in eggs and cell cultures. These are our primary concern and a discussion of evolution must be based on the assumption that knowledge painstakingly obtained with established strains applies to them. As our work progresses, this assumption must be continuously challenged.

The size of the genome

Sarov and Becker (1969) isolated the deoxyribonucleic acid (DNA) of the TE-55 strain of *C. trachomatis* by a procedure which minimized shearing and yielded a large number of cyclic forms. Electron microscopic measurements and density gradient determinations indicated that an unsheared molecule of DNA contains about 11×10^5 nucleotide pairs. A comparison of this value with those obtained by others with chlamydiae and other bacteria and with viruses is illustrated in Table I. Kingsbury (1969) obtained somewhat lower values for two strains of *Chlamydia*, but his method was entirely different. It was based on the finding by Britten and Kohne (1968) that the rate of reassociation between single strands of

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TABLE I

Size of bacterial and viral chromosomes

Organism	10 ⁶ nucleo- tide pairs	Method ^a	Reference
<i>Escherichia coli</i> ^b	45	AR	Cairns (1963)
<i>Neisseria catarrhalis</i>	23	RK	Kingsbury (1969)
<i>N. meningitidis</i>	17	RK	Kingsbury (1969)
<i>Rickettsia rickettsii</i>	15	RK	Kingsbury (1969)
<i>Chlamydia psittaci</i> (MN)	8.5	RK	Kingsbury (1969)
<i>C. trachomatis</i> (MRC-1/G)	6	RK	Kingsbury (1969)
<i>C. trachomatis</i> (TE-35)	11	EM, DG	Sarov and Becker (1969)
<i>Mycoplasma hominis</i>	2.5	EM	Bode and Morowitz (1967)
Vaccinia virus	2.5	EM, DG	Sarov and Becker (1967)
			Becker and Sarov (1968)
Coliphage T2 ^b	1.4	AR	Cairns (1963)
Polyoma virus	0.05	EM	Weil and Vinograd (1963)

^a AR = autoradiography; RK = reassociation kinetics; EM = electron microscopy; DG = density gradient.

^b Reference chromosomes.

bacterial DNA fragments is inversely proportional to the size of the genome. Among the bacteria studied, the spread in size of the DNA is about 8-fold, but there is no overlap with the larger viruses. The spread among double-stranded DNA viruses is at least 50-fold. The DNA of chlamydiae are among the smaller bacterial DNA, but not necessarily the smallest. The experiments of Bode and Morowitz (1967) are entirely comparable to those of Sarov and Becker (1969) and it is apparent that the genome of the agent of trachoma is at least as large as that of *Mycoplasma hominis*, which, of course, is capable of multiplication without the support of host cells. Thus, chlamydiae have sufficient DNA to account for a multitude of functions. Their inability to grow independently of host cells can best be attributed to adaptation to an intracellular environment, rather than to extensive deletions in their genomes.

Functions reflecting adaptation to intracellular environment

Cell wall. Tamura, Manire, and their associates (Tamura and Manire, 1967; Tamura *et al.*, 1967; Manire and Tamura, 1967; Matsumoto and Manire, 1970) isolated reticulate bodies of the MN agent, relatively uncontaminated with dense particles (elementary bodies) and were able to carry out a series of studies on each form. Both types of cells were shown to have trilaminar cell walls, but those of the reticulate bodies lacked rigidity and were more easily disrupted by sonic treatment and trypsin. Tribby (1970) recently obtained good evidence that the cell walls of both forms contain peptidoglycans, but the reticulate bodies are less rigid because the peptidoglycans are not cross-linked by peptide bridges. This finding in part unravels the mystery of the developmental cycle. In a suitable

intracellular environment, the peptide bridges containing D-alanine are dissolved, presumably by the elementary-body, as one of the first steps in reticulate body formation. For some unknown reason this situation favors greater exchange of nutrients with the host cell. After chlamydial cell division is about completed, the cross-linkages are regenerated and reticulate bodies are converted into elementary bodies.

Metabolic activity of isolated resting cells. Technical difficulties have limited metabolic studies (Weiss *et al.*, 1964, 1968; Weiss, 1965, 1967; Moulder *et al.*, 1963; Vender and Moulder, 1967; Weiss and Wilson, 1969) almost exclusively to the elementary body, although the reticulate body is undoubtedly the more active form. The salient features of the metabolism of intact isolated elementary bodies, which are analogous to the resting cells of other systems, can be summarized as follows:

- (a) Among the substrates metabolized are glucose-6-phosphate, 6-phosphogluconate, pyruvate, glutamate, α -ketoglutarate, aspartate, and isoleucine.
- (b) The rates are one to two orders of magnitude lower than those of most other bacteria utilizing the same substrates.
- (c) The reactions in most, but not all, cases involve one or a few enzymes, rather than a series, are anaerobic, and result in the evolution of CO_2 . The MN agent is also able to incorporate carbon from glucose-6-phosphate, aspartate and isoleucine into lipid, primarily into the fatty acids of phosphatidyl ethanolamine and phosphatidyl choline (Weiss and Wilson, 1969; Gaugler *et al.*, 1969).
- (d) There is no evidence that useful energy is derived from any of these reactions. In fact, several of these reactions require exogenous adenosine triphosphate (ATP), a rare if not unique requirement for intact microorganisms.
- (e) Several reactions require or are greatly stimulated by pyridine nucleotides, again an unusual event for intact cells. Electron transport systems appear to be incomplete. There is no evidence of the presence of functioning endogenous cytochromes, but the microorganisms can reduce added cytochrome C in the presence of either reduced pyridine nucleotides or a combination of an oxidized pyridine nucleotide plus a substrate that elicits its reduction (Allen and Bovarnick, 1957; L. A. Kiesow, personal communication).

The above-described reactions can be interpreted as the vestige or more extensive activities that can take place either (a) in concert with host cell constituents or (b) in reticulate bodies and are repressed in elementary bodies. Evidence for the first possibility is of a negative nature. If the chlamydial cell takes advantage of what it finds in the host cell, it must retain strictly procaryotic activities and at least two of the above-described reactions are procaryotic. Aspartic acid is metabolized via diaminopimelic acid to lysine (Moulder *et al.*, 1963; Weiss and Wilson, 1969). Makino *et al.* (1970) have shown that chlamydial cells have branched fatty acids not encountered in avian or mammalian cells, and lipid synthesis may preferentially involve these compounds. On the positive side, the chlamydial cell

may well depend on the host cell for a number of energy-rich intermediates and for the reoxidation of reduced pyridine nucleotides. The second possibility, namely that reticulate bodies are metabolically more active than elementary bodies, is supported by the experiments of Tamura and Manire (1968) who showed that the cytochrome C reductase activity of reticulate bodies was far greater than that of the elementary bodies and that this was in part due to greater permeability.

Most of these experiments were done with the MN agent, but many were repeated with strains of *C. trachomatis*. Qualitative differences in metabolic patterns have not been encountered.

Interaction with the host cell. The chlamydial cell has no active mechanism of penetration and this is not surprising, since it is not capable of generating ATP. To gain entrance into a host cell, it must depend on the phagocytic activity of the host (Friis, 1971). As a result, it is taken up in a vacuole and the developmental cycle takes place there. How the chlamydial cell avoids the release or action of lysosomal enzymes is not known. Although it is not unusual for a microorganism to achieve an intracellular location by phagocytosis, other mechanisms have been described. Rickettsiae, for example, actively penetrate into host cells, since penetration closely parallels their ability to oxidize glutamate and to produce ATP (Cohn *et al.*, 1959; Bovarnick and Schneider, 1960). By this method they gain access into the cytoplasm of the cell and are not enclosed in a vacuole (Anderson *et al.*, 1965).

The chemical events that accompany the intracellular developmental cycle of chlamydiae have been elucidated in Moulder's laboratory. A major tool was the inhibitor, cycloheximide, which prevents protein and DNA syntheses of eucaryotic but not of procaryotic cells. By the use of this tool Alexander (1968, 1969) showed that chlamydiae synthesize their own DNA and protein. Lin (1968) showed that as chlamydiae begin their syntheses they inhibit host thymidine kinase activity. Host DNA synthesis declines and reduction in protein and RNA syntheses follow. Moulder (1970) has shown that glucose metabolism proceeds at the same rate in multiplying and non-multiplying cells, and that except for a rise in aerobic glycolysis, the catabolism is not greatly affected by infection with the MN agent. The above-described findings provide evidence for Moulder's hypothesis that the energy of the host is diverted from production of its own protein and DNA to that of the chlamydial cells.

The three characteristics of chlamydiae, inability to generate its own ATP, inhibition of host DNA synthesis, and dependence on energy that becomes available as a consequence of this inhibition, represent an exquisite adaptation to an intracellular environment. For instance, if chlamydiae produced significant amounts of ATP, the rates of syntheses could quickly exceed the capacity of the host cell and the intracellular environment could be destroyed. If host DNA synthesis were not inhibited, competition with the host cell for essential metabolites would stifle chlamydial metabolism.

Of course, this is not the only successful combination of properties for intracellular growth, but in any case the host cell must not be destroyed too quickly. Rickettsiae generate some of their own ATP, but do not prematurely exhaust the

host's nutrients because they multiply slowly, with a mean doubling time of 6 hours or longer. It was shown by Dasinger and Wilson (1962) that a virulent strain of *Brucella abortus* oxidizes glutamate at about half the rate of an avirulent strain. This is possibly related to the finding by Freeman *et al.* (1964) that avirulent brucellae cannot be cultivated in guinea pig monocytes, not because they do not have the ability to grow in them, but because they destroy them too rapidly.

Variation in metabolic activity among strains of Chlamydia. The numerous observations made on strains other than MN and a few other established agents have not provided evidence that there are major exceptions to the metabolic pattern just outlined. The criteria used to separate *C. trachomatis* from *C. psittaci* (Page, 1968) indicate that some important differences do exist, but each one involves a relatively small series of enzymes. The morphology of the inclusion is difficult to evaluate in biochemical terms, unless the compact inclusion of *C. trachomatis* is due to the deposition of glycogen. For a microorganism that has an active glucose metabolism, the ability - or lack of it - to synthesize, store, and degrade glycogen represents a difference of a few enzymes. Sulfadiazine-susceptible strains synthesize folic acid from simple components. Those which are resistant convert dietary folic acid to their own specific form of the vitamin (Colón, 1962). D-cycloserine inhibits two enzymes involved in alanine metabolism leading to the formation of the peptide bridges of the cell wall. Differences in susceptibility to this inhibitor between the two species of *Chlamydia* are a matter of degree and are best interpreted as being due to different affinities of the enzymes for alanine and D-cycloserine (Moulder *et al.*, 1965). It can be concluded that the study of metabolism emphasizes the characteristics that chlamydiae have in common, rather than those which separate them.

DNA heterogeneity among strains of Chlamydia

The base composition of the DNA of strains of *Chlamydia* was studied in several laboratories (Moulder, 1966; Kingsbury and Weiss, 1968; Gerloff *et al.*, 1970). There appears to be a small but significant difference between the base compositions of *C. psittaci* and *C. trachomatis*. In one study (Weiss *et al.*, 1970) the molar percentages of guanine plus cytosine (% G + C) were 39 and 42, respectively. It is likely, as suggested by Gerloff *et al.* (1970) that intermediate values will be found as the investigations are extended to a larger number of strains.

The difference in base ratios between the two species is reflected in the results obtained in DNA reassociation experiments (Kingsbury and Weiss, 1968; Weiss *et al.*, 1970). The single-stranded DNA of one species was immobilized on membrane filters and incubated with DNA fragments labeled with carbon-14 derived from another species. The degree of reassociation occurring in moderately high salt concentration (0.6 M NaCl) and at a temperature of 67°C was compared to the reaction occurring with homologous fragments of labeled DNA. (The term 'reassociation' refers to heterologous as well as homologous duplex formation.) The combination of salt concentration and temperature used in these investigations can be called 'semi-restrictive' or not requiring a high degree of homogeneity between DNA strands. It was a surprise, therefore, that DNA reassoci-

TABLE II

Similarity among the DNA of human isolates of C. trachomatis^a

Isolate	Per cent G + C	Per cent reassociation with MRC-1/G
MRC-1/G	42.2	(100;
TW-3	42.1	95
Lgv (JH)	42.7	97
TE-55		97
Cal 1		96

^a From Kingsbury and Weiss, 1968; Weiss *et al.*, 1970.

ation between *C. psittaci* and *C. trachomatis* was only 10% of the homologous reaction. This small amount of reassociated DNA was dissolved at relatively low temperatures, indicating that there were frequent gaps in its base pairing.

Exactly the opposite results were obtained when reassociation experiments were carried out between two strains of *C. psittaci* or between any two of the five human isolates of *C. trachomatis* listed in Table II. The degree of reassociation amounted to 95-97%, and the reassociated DNA were about as thermostable - or as extensively paired - as those obtained with two single strands of the same organism. Comparable levels of reassociation were obtained earlier between *C. psittaci* strains by Gerloff *et al.* (1966).

The degree of homogeneity within a species is tested, however, by comparing a prototype strain with one which fulfils the criteria for inclusion in the species, but is otherwise quite dissimilar. The agent of mouse pneumonitis (MoPn) plays such a role in the species *C. trachomatis*. It was shown (Weiss *et al.*, 1970) that it is more closely related to *C. trachomatis* than to *C. psittaci*. Like other strains of *C. trachomatis*, MoPn has a base ratio of 42% G + C and reassociates with *C. psittaci* to the extent of 15%. However, the degree of reassociation with the MRC-1/G strain is 63% under semi-restrictive conditions (67°C) and 29% under highly restrictive conditions (75°C). The thermostability of the MRC-1/G-MoPn duplexes is low. While the homologous duplexes or those formed between the two human strains of *C. trachomatis* separate at the temperature of denaturation of native DNA, the midpoint of separation of the MRC-1/G-MoPn duplexes is about 11 to 13°C lower - an unusually low thermostability for two strains able to reassociate to an appreciable extent. Thus, these studies have demonstrated that there is a certain amount of similarity between polynucleotide sequences of the two DNA, but have failed to show identity in any part of their genomes.

The results obtained with *Chlamydia* have their parallel in other bacteria. For example, Kingsbury (1967) has shown that the degree of similarity in polynucleotide sequence of the DNA of 10 strains of *Neisseria meningitidis* is of the order of 95-100%. These strains are all potential human pathogens, but differ

greatly in antigenic specificity. On the other hand, we have shown more recently (Weiss *et al.*, 1971) that strains of *N. lactamica*, which are isolated from man but are not associated with disease, have a much lower level of similarity to *N. meningitidis* or to each other in polynucleotide sequence. If these two examples have general significance, we may surmise that human pathogens are classified more narrowly than other microorganisms.

CONCLUSIONS

There are essentially three methods by which we can judge evolution in a group of microorganisms. The first one - discussed extensively in other papers - is antigenic analysis, which detects the most minute differences. If applied to intact cells it emphasizes changes in surface structures that might have emerged as the result of the selective pressures of antibodies. Probably, it also emphasizes the more recent changes. From the practical point of view of strain identification, epidemiological investigation, or vaccine production, it is the most useful method.

The second is the study of the DNA. This method is not sufficiently sensitive to detect genetic changes affecting antigenic structure, degrees of virulence for man or animals, smooth to rough variation, and other changes that involve less than 5% of the genome. It is best adapted to serve as a guide to define the species. In this role it also serves a very useful purpose. In the case of *C. trachomatis*, studies carried out thus far suggest that genetic differences among the human pathogens are relatively small. In attempts to understand the variegated aspects of human disease caused by *C. trachomatis* it encourages us to pay more attention to host and environmental factors. Of course, this is a highly biased view derived from a study with a few strains well adapted to yield large harvests in the laboratory. It must be tested against the strains that have been more directly associated with disease.

The MoPn agent is perhaps a good example of the many strains that can be isolated from healthy animals simply by passing suspensions from organs from animal to animal in series. The art of DNA reassociation has not advanced to the point that it can analyze the relatedness of two DNA that reassociate extensively, but form duplexes that are entirely thermolabile. Therefore, what follows is entirely speculative. The differences between the MoPn and the human strains of *C. trachomatis* might well be the result of genetic 'drift', or residence in different hosts for a sufficiently long time to allow the emergence of numerous point mutations. Some of these mutations may not have affected the function of the genes involved, since the triplet code allows a certain amount of variation. Others may have produced minor changes affecting the interaction of the microorganism with the host cell that are not easily detected in the laboratory. The survival of many of these changes may not have been due as much to selective advantage as to the state of isolation of these strains in certain hosts. The time required for the establishment of this diverging evolution was determined primarily by two factors. One is the extent of growth of the microorganisms in the new host, which might not have been the same during the entire course of this evolution. The second is the ratio of lethal to total mutations, which might not be the same in the intracellular and extracellular environments. In any case, it is a good assumption that

TABLE III

Hypothetical steps in the evolution of Chlamydia

Step	Function selected	Selective advantage
1	Survival after phagocytosis	Entrance into host cell
2	Loss of cross-linkages of cell wall	Greater interaction with host cell
3	Inhibition of host DNA synthesis	Less competition with host cell requirements
4	Loss of ATP synthesis, dependence on host energy	Rate of growth adjusted to environment
5	Reformation of cross-linkages of cell wall	Greater stability in extracellular environment

the common ancestor of agents such as MoPn and the human strains of *C. trachomatis* is relatively remote.

The difference in DNA between *C. trachomatis* and *C. psittaci* is so great that it is difficult to see any evolutionary relationship between the two species. We cannot ignore, however, the third and best established method of analyzing evolutionary history, namely the study of structure and function. This method is best adapted to the definition of the genus. The similarity of all chlamydiae in morphology and in mechanisms by which they have become adapted to an intracellular environment is striking. This is particularly obvious when they are compared to such obligate or facultative intracellular bacteria as rickettsiae, mycobacteria, or brucellae. The common ancestor of *C. trachomatis* and *C. psittaci* must be quite remote, however, to account for their difference in polynucleotide sequence. The two species retained their similarity possibly because the appearance of one change in a population greatly favored the emergence of a particular second change, and so on in succession. Thus, two related but not necessarily identical microorganisms undergoing an initial similar mutation continued to acquire similar functions, while genotypically they 'drifted' further apart. An example of such a series of changes - which is highly speculative - is illustrated in Table III. Successful entrance into a host cell by phagocytosis favors the mutants that can dissolve the cross-linkages of the cell wall and interact more freely with the host cell. Selective inhibition of host cell synthesis is the next step to give such an organism an overwhelming advantage. Loss of ability to produce its own ATP and dependence on energy derived from the host is further insurance that the micro-environment will not be destroyed too rapidly. The ability to reform the cross-linkages is a necessary function to protect the microorganism in its journey from cell to cell and from host to host.

The above presentation of the biology and evolution of *Chlamydia* is perhaps an oversimplification. Experience teaches us, however, that as we come closer to the understanding of a phenomenon, the explanations become simpler.

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